Mechanisms for transcellular diapedesis: probing and pathfinding by 'invadosome-like protrusions'

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Summary

Immune-system functions require that blood leukocytes continuously traffic throughout the body and repeatedly cross endothelial barriers (i.e. diapedese) as they enter (intravasate) and exit (extravasate) the circulation. The very earliest studies to characterize diapedesis directly in vivo suggested the coexistence of two distinct migratory pathways of leukocytes: between (paracellular pathway) and directly through (transcellular pathway) individual endothelial cells. In vivo studies over the past 50 years have demonstrated significant use of the transcellular diapedesis pathway in bone marrow, thymus, secondary lymphoid organs, various lymphatic structures and peripheral tissues during inflammation and across the blood-brain barrier and blood-retinal barrier during inflammatory pathology. Recently, the first in vitro reports of transcellular diapedesis have emerged. Together, these in vitro and in vivo observations suggest a model of migratory pathfinding in which dynamic 'invadosome-like protrusions' formed by leukocytes have a central role in both identifying and exploiting endothelial locations that are permissive for transcellular diapedesis. Such 'probing' activity might have additional roles in this and other settings.

This article is part of a Minifocus on invadopodia and podosomes. For further reading, please see related articles: 'Invadosomes at a glance' by Stefan Linder (*J. Cell Sci*. **122**, 3009-3013), 'Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia' by Renaud Poincloux et al. (*J. Cell Sci*. **122**, 3015-3024) and 'Actin machinery and mechanosensitivity in invadopodia, podosomes and focal adhesions' by Corinne Albiges-Rizo et al. (*J. Cell Sci*. **122**, 3037-3049).

Key words: Leukocyte, Endothelium, Diapedesis, Podosome, Invadopodia, Invadosome, Migration, Transcellular

Introduction

To fulfill their roles of immune surveillance and pathogen elimination, cells of the immune system (such as blood leukocytes, which include lymphocytes, monocytes, dendritic cells and neutrophils) must continuously traffic throughout the body (von Andrian and Mackay, 2000). This requires not only locomotion and chemotaxis, but also an explicit propensity to negotiate and cross tissue barriers. Such 'migratory pathfinding' represents an important and rate-limiting aspect of leukocyte trafficking, and is considered a key therapeutic target for inflammatory and immune-mediated disease (Ley et al., 2007; von Andrian and Mackay, 2000).

Leukocyte trafficking can be broken into two major phases: movement within the vascular and lymphatic circulation, and migration within tissues. The vascular and lymphatic circulatory systems are lined by monolayers of endothelial cells that grow on an ablumenal layer of extracellular matrix (the basement membrane); these cells form organized intercellular junctional zones that include adherens junctions, tight junctions and gap junctions (Baluk et al., 2007; Bazzoni and Dejana, 2004; Pepper and Skobe, 2003). In this way, the endothelium serves as the principal (selectively permeable) barrier between the circulation and the underlying tissues. Each phase change during trafficking [i.e. movement into (intravasation) or out of (extravasation) the circulation] therefore requires that leukocytes cross the endothelium (diapedese) (Fig. 1).

The overall process of leukocyte extravasation has been intensively studied, whereas that of intravasation remains poorly understood. Extravasation of circulating leukocytes is initiated by largely selectin-dependent transient rolling-type interactions on the lumenal surface of the endothelium (Luscinskas et al., 1994; Springer, 1994) (Fig. 1B). This facilitates chemokine-dependent activation of leukocytes, which in turn triggers firm adhesion that is mediated by the binding of leukocyte integrins (such as LFA1, Mac1 and VLA4) to their endothelial ligands [such as intercellular adhesion molecule (ICAM)1 and 2, and vascular cell adhesion molecule 1 (VCAM1)] (Fig. 1B) (Carman and Springer, 2003; Luo et al., 2007). Subsequently, lymphocytes undergo actin-dependent polarization and integrin-dependent lateral migration on the lumenal surface of the endothelium; this movement seems to allow them to seek out sites that are permissive for diapedesis (Phillipson et al., 2006; Schenkel et al., 2004) (Fig. 1B; Fig. 2). Formally, the basic steps for intravasation should include interstitial migration towards the vessel, migration across the basement membrane and endothelial barriers (diapedesis), and, ultimately, release of lumenal leukocytes into the circulation (Fig. 1A). It is now appreciated that diapedesis, whether it occurs during intravasation or extravasation, can occur by two distinct routes or pathways: either via disassembly of the intercellular junction to form a paracellular gap (paracellular diapedesis) or via the formation of a transcellular pore directly through an individual endothelial cell (transcellular diapedesis).

The concept of one cell passing through or, in effect, entering another cell (as occurs in transcellular diapedesis) might seem a bizarre and unlikely one. In fact, over the past ~100 years, a wide range of such 'cell-in-cell' interactions have been documented (Overholtzer and Brugge, 2008). A large number of in vitro and in vivo studies have demonstrated that a variety of viable (i.e. non-

apoptotic) blood leukocytes are internalized into epithelial, liver, Kupffer, follicular-dendritic and thymic nurse cells, as well as into neurons, astrocytes, megakaryocytes and several kinds of neoplastic cells (Overholtzer and Brugge, 2008). With respect to transcellular diapedesis specifically, ~45 studies have been published demonstrating significant use of the transcellular route in a wide range of in vivo settings [recently reviewed in detail (Sage and Carman, 2009) and summarized below (Table 1)]. Thus, the premise that cells move into and through other cells is, indeed, broadly relevant. However, the lack of clear evidence for transcellular diapedesis in initial studies using cultured in vitro endothelial models (Beesley et al., 1979; Burns et al., 2003; Furie et al., 1987; Luscinskas et al., 2002; Muller, 2003; Pawlowski et al., 1988) seemed to largely preclude widespread acceptance, as well as mechanistic investigation, of this pathway.

Recently, the first in vitro observations of transcellular diapedesis have been made. Here, we discuss these alongside previous in vivo studies, and focus on the emerging roles for invadosome-like protrusions (ILPs) in transcellular diapedesis. In addition, we consider distinct settings for transcellular diapedesis and additional roles for the probing activity of ILPs. Mechanisms for paracellular

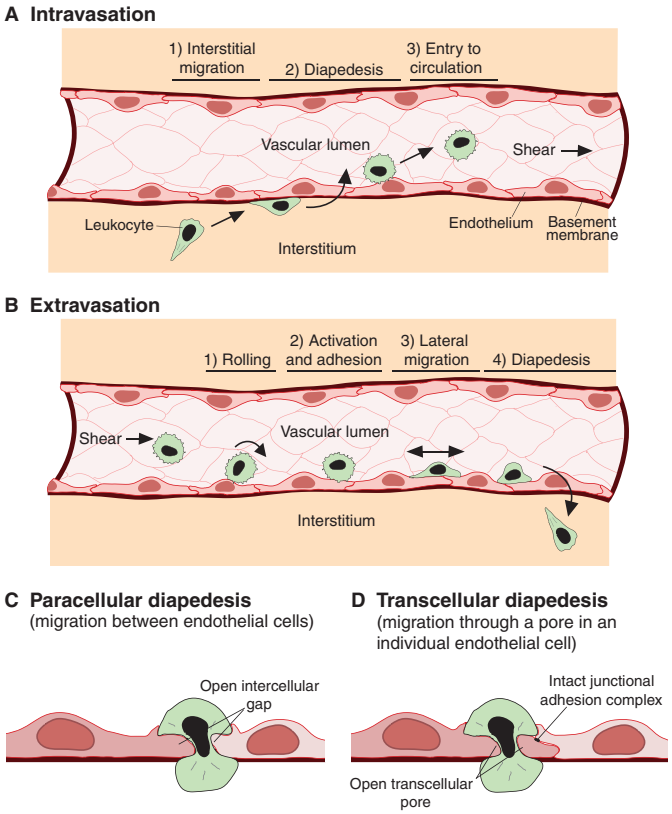

Fig. 1. Paracellular and transcellular routes of leukocyte diapedesis during intravasation and extravasation. Trafficking of leukocytes throughout the body requires their movement into (intravasation) and out of (extravasation) the vascular and lymphatic circulation. (A) Stages of intravasation. Details of the process by which leukocytes (green) enter vascular structures (exemplified here as a post-capillary venule; pink) and lymphatic structures are not well characterized. In general, this is thought to involve steps of interstitial migration towards the vessel, migration across the basement membrane and endothelial barriers (diapedesis), and, ultimately, release of lumenal leukocytes into circulation. (B) Stages of extravasation. The leukocyte extravasation process has been intensively studied and shown to involve, in the first instance, transient rolling-type interactions mediated predominantly by selectins (Springer, 1994). This facilitates chemokine-dependent activation and firm arrest, which is mediated by the binding of leukocyte integrins (e.g. LFA1, Mac1 and VLA4) to endothelial cell-adhesion molecules (e.g. ICAM1, ICAM2 and VCAM1). Subsequently, leukocytes migrate laterally over the surface of the endothelium, probing for a site to penetrate through it (see also Fig. 2). Finally leukocytes cross the endothelial barrier (diapedese) and enter the interstitium. (C,D) The process of diapedesis, whether during intravasation or extravasation, can occur by two distinct pathways: paracellular or transcellular. (C) Paracellular diapedesis. Leukocytes and endothelial cells coordinately disassemble endothelial cell-cell junctions and open up a gap between two or more endothelial cells (Muller, 2003). (D) Transcellular diapedesis. Leukocytes migrate directly through individual endothelial cells via a transient transcellular pore that leaves endothelial cell-cell junctions intact. Note that the two individual endothelial cells in C and D are distinguished by different shades of pink.

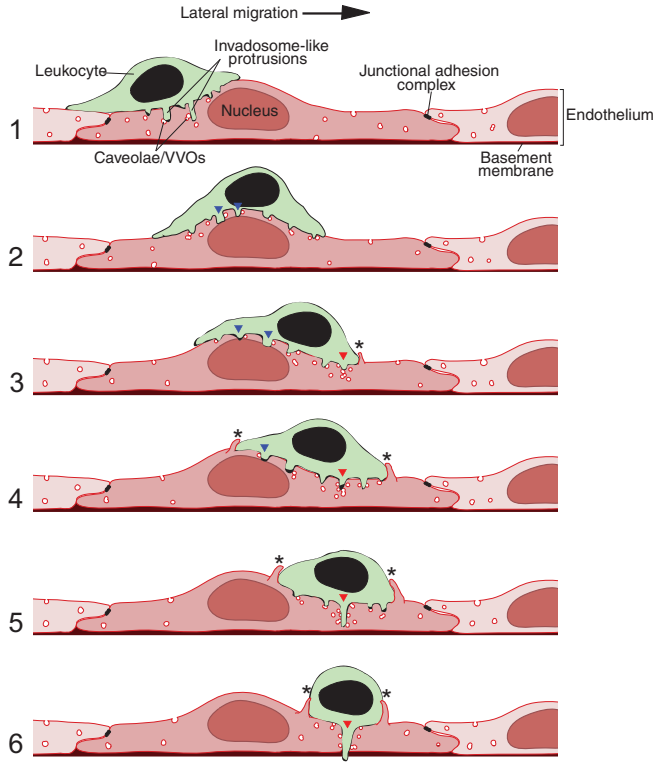

Fig. 2. Leukocytes probe the endothelium with ILPs. A leukocyte (green) is depicted in the process of lateral migration over, and transcellular diapedesis through, an endothelial monolayer (pink). Numbered panels show successive time points that are intended to represent intervals of ~30-60 seconds. Dynamic insertion (to ~0.2-1 μm in depth) of multiple ILPs into the apical surface of the endothelium, and their retraction, is shown as lateral migration proceeds (panels 1-4). ILPs that form over the nucleus of the endothelial cell are impeded by physical resistance from the nuclear lamina and remain shallow (blue arrowheads in panels 2-4; see also ultrastructural equivalent in Fig. 3B). At a location of sufficiently low endothelial resistance, an ILP progressively extends several μm in depth, ultimately breaching the endothelium transcellularly (red arrowheads in panels 3-6; see also ultrastructural equivalent in Fig. 3D). Intracellular vesicular structures that are similar to caveolae and VVOs are enriched near, and fused to, endothelial invaginations ['podo-prints' (Carman et al., 2007)] that are formed by leukocyte ILPs (see also Fig. 3A,C). Also depicted is the previously described 'transmigratory cup' structure (asterisks), which consists of vertical endothelial microvillus-like projections [rich in actin, ICAM1, VCAM1, PECAM1 and JAM1 (Barreiro et al., 2002; Carman et al., 2003; Carman and Springer, 2004)] that surround the periphery of adherent leukocytes. This structure seems to guide diapedesis and to provide a vertical traction substrate for the protrusion of leukocytes against the endothelial surface.

Table 1. In vivo observations of transcellular diapedesis

Tissue or structure[1]	Leukocytes	Stimulus[2]	Species	References[3]
Bone marrow	Eosinophils, granulocytes, lymphocytes	Untreated, eotaxin, IL5	Guinea pig, mouse, rat	(**Becker and De Bruyn, 1976; Campbell, 1972; Chamberlain and Lichtman, 1978; De Bruyn et al., 1971; Muto, 1976**; Palframan et al., 1998; Wolosewick, 1984)
Thymus	Lymphocytes	Untreated	Guinea pig, rat	(Toro and Olah, 1967; **Ushiki, 1986**)
Lymph-node HEV (a, c, m, p, pa)	Lymphocytes	Untreated, *Staphylococcus aureus*	Guinea pig, hamster, mouse, rat	(**Cho and De Bruyn, 1979; Cho and De Bruyn, 1981; Cho and De Bruyn, 1986; Farr and De Bruyn, 1975; Marchesi and Gowans, 1964**)
Peyer's patch HEV	Lymphocytes	Untreated, intestinal irritant	Guinea pig, mouse, rat	(**Azzali et al., 2008; Cho and De Bruyn, 1986; Yamaguchi and Schoefl, 1983**)
Tonsil HEV	Lymphocytes	Tonsillitis	Human	(Indrasingh et al., 2002)
Lymphatic structures (ALPA, ALV, lymphatic sinusoid, SI-lacteals, TAAL)	Lymphocytes, macrophages, neutrophils	Untreated, lymphatic stasis, prolonged fast	Chicken, mouse, rabbit, rat	(**Azzali, 1990; Azzali, 1998; Azzali, 2007a; Azzali and Arcari, 2000; Azzali et al., 1990a; Azzali et al., 1990b; Farr et al., 1980**; Olah and Glick, 1985)
Mesentery	Eosinophils, lymphocytes, monocytes, neutrophils	Mechanical trauma	Rat	(Marchesi and Florey, 1960)
Pancreas	Leukocytes	Ischemia	Dog	(Williamson and Grisham, 1960; Williamson and Grisham, 1961)
Skin	Eosinophils, neutrophils	C5a, fMLP, NAP, IL1, LTB4	Guinea pig, human, mouse	(**Feng et al., 1998; Hoshi and Ushiki, 1999**; Schubert et al., 1989)
Liver, lung, spleen, kidney, heart	Lymphocytes	IL2	Mouse	(Fujita et al., 1991)
Cremaster	Neutrophils	MIP2	Mouse	(**Phillipson et al., 2006; Phillipson et al., 2008**)
Blood-brain barrier	Lymphocytes, neutrophils	α-bungarotoxin, EAE, EAN, post-operative, thalamus degeneration	Cat, mouse, rabbit, rat	(Astrom et al., 1968; Barron et al., 1974; **Faustmann and Dermietzel, 1985; Lossinsky et al., 1989; Lossinsky et al., 1991**; Matthews and Kruger, 1973; Raine et al., 1990; **Wolburg et al., 2005**)
Blood-retinal barrier	Granulocytes, lymphocytes, macrophages	EAU, IL1	Rat	(Bamforth et al., 1997; **Greenwood et al., 1994**)

[1]a, axillary; ALPA, absorbing lymphatic peripheral apparatus; ALV, absorbing lymphatic vessel; c, cervical; p, popliteal; pa, para-aortic; m, mesenteric; SI, small intestine; TAAL, tumor-associated absorbing lymphatic. [2]C5a, complement component 5a; EAE, experimental autoimmune encephalomyelitis; EAN, experimental autoimmune neuritis; EAU, experimental autoimmune uveoretinitis; fMLP, formyl-Met-Leu-Phe; IL, interleukin; LTB4, leukotriene B4; MIP2, macrophage inflammatory protein 2; NAP, neutrophil-activating peptide. [3]Studies providing at least 'reasonably conclusive' or unequivocal demonstration of transcellular diapedesis (via either SEM, serial-section TEM, or serial-section confocal fluorescence microscopy in the presence of junctional markers) are highlighted in bold.

diapedesis have been reviewed extensively elsewhere (Burns et al., 2003; Ley et al., 2007; Luscinskas et al., 2002; Muller, 2001; Muller, 2003) and will not be covered here in detail.

In vivo settings for transcellular diapedesis

An overview of leukocyte trafficking

As noted above, immune cells exhibit the distinct ability and requirement to traffic throughout almost all recesses of the body (von Andrian and Mackay, 2000). Leukocytes originate in the bone marrow, where they begin their life cycle by migrating into the bloodstream. T and B lymphocytes then enter and exit various lymphoid organs (including the thymus, lymph nodes, Peyer's patches, spleen and tonsils) as part of their maturation processes and immune-surveillance functions. Monocytes constitutively migrate from the circulation into the peripheral tissues, where they differentiate into antigen-presenting cells (APCs), including macrophages and dendritic cells. These, in turn, traffic out of the tissue and into the lymphatic system through afferent lymphatic vessels that carry them to secondary lymphoid organs. In cases of infection, APCs bearing pathogen-derived antigen interact with and activate the expansion of antigen-specific lymphocytes. These lymphocytes then differentiate into effector or memory lymphocytes, which enter the vascular circulation and join innate immune cells (such as neutrophils) in migrating into the infected or inflamed peripheral tissues. Finally, several recent studies have begun to document so-called 'reverse transmigration', whereby inflammatory leukocytes are thought to leave the peripheral tissues (during the resolution phase of inflammation) by reversing their migratory path and undergoing intravasation to re-enter the vascular circulation (Huttenlocher and Poznansky, 2008).

The diapedesis associated with each of the above trafficking steps can occur in vastly different tissues and involve distinct endothelia (Aird, 2007a; Aird, 2007b), leukocyte subtypes and migration stimuli. Thus, it should not be surprising that the existing studies suggest wide variation in the extent to which para- and transcellular diapedesis routes are used in the various in vivo settings examined, as outlined below. The majority of in vivo reports of transcellular diapedesis have been conducted using methods that are appropriate for reasonably conclusive, and often unequivocal, assessment of the route of diapedesis [e.g. scanning electron microscopy (SEM), serial-section transmission electron microscopy (TEM), or serial-section confocal fluorescence microscopy in the presence of junctional markers; see highlighted references in Table 1]; however, it is important to note when discussing such studies that some have

employed single-section TEM only, which cannot conclusively determine the route of diapedesis.

Transcellular diapedesis in bone marrow

Bone marrow is the primary locus for hematopoiesis. Thus, the first requirement for essentially all leukocytes is to enter the circulation by diapedesis across the bone-marrow endothelium. Electron microscopy (EM) studies of such diapedesis provide largely conclusive and consistent evidence for the predominant use of the transcellular pathway by both mononuclear cells and granulocytes (Becker and De Bruyn, 1976; Campbell, 1972; Chamberlain and Lichtman, 1978; De Bruyn et al., 1971; Muto, 1976; Palframan et al., 1998; Wolosewick, 1984) (Table 1). These diapedesis events occurred predominantly via transcellular pores that formed in close juxtaposition to intact intercellular junctions ('peri-junctionally'), where the endothelium is generally most attenuated (Campbell, 1972; Chamberlain and Lichtman, 1978; De Bruyn et al., 1971; Wolosewick, 1984). Interestingly, initiation of transcellular diapedesis in this setting was also shown to involve actin-rich leukocyte protrusions that have been termed 'podosomes' (discussed below) (Wolosewick, 1984).

Transcellular diapedesis in the thymus

T lymphocytes initially enter the bloodstream as T-cell progenitors, which rapidly home to the thymus in order to undergo maturation before re-entering the circulation. Although lymphocyte intravasation from the thymus has not been extensively studied, two investigations that used combined SEM and TEM have demonstrated that it occurs via transcellular diapedesis (Toro and Olah, 1967; Ushiki, 1986).

Transcellular diapedesis in secondary lymphoid organs

Secondary lymphoid organs provide a highly organized nexus for information exchange between naive and memory lymphocytes and APCs, thereby allowing efficient activation of antigen-specific lymphocytes (Bajenoff et al., 2007). Lymphocytes constitutively traffic to secondary lymphoid organs by extravasating across specialized high endothelial venules (HEVs). HEVs are distinguished by their thick, cuboidal endothelial phenotype and their constitutive expression of chemokines (such as CCL19 and CCL21) and adhesion molecules [such as peripheral node addressin (PNAd), ICAM1, ICAM2 and mucosal addressin cell adhesion molecule 1 (MAdCAM1)] that are necessary for recruitment of naive and memory lymphocytes (Miyasaka and Tanaka, 2004).

The relative role of para- and transcellular pathways in the HEVs of secondary lymphoid organs is particularly unclear and controversial. Studies of cervical, mesenteric, popliteal, para-aortic, axillary and tibial-popliteal lymph nodes in a range of animal models (rat, mouse, hamster, guinea pig and chicken) have reported predominant use of transcellular diapedesis (Cho and De Bruyn, 1979; Cho and De Bruyn, 1981; Cho and De Bruyn, 1986; Farr and De Bruyn, 1975; Marchesi and Gowans, 1964). Diapedesis in Peyer's patches (in rat, mouse and guinea pig) was seen to occur either by a transcellular pathway exclusively (Azzali et al., 2008; Cho and De Bruyn, 1986) or by concomitant transcellular and paracellular modes (Yamaguchi and Schoefl, 1983). In HEVs of tonsils removed from humans with tonsillitis, HEVs were crossed both paracellularly and transcellularly (Indrasingh et al., 2002). Other studies (not reviewed here) in various secondary lymphoid organs report exclusive use of paracellular diapedesis.

Transcellular diapedesis in lymphatic structures

Afferent lymphatic vessels (the blind-ending lymphatic vessels that are distributed throughout the peripheral tissues) represent the main points for entry of APCs into secondary lymphoid organs. Contrary to earlier views, recent studies have shown that endothelium of afferent lymphatic vessels are joined by continuous cell-cell adhesions, which include zones preferentially enriched in either VE-cadherin or PECAM-1 (Baluk et al., 2007), and that leukocyte intravasation at this locus is an active adhesion-molecule- and chemokine-dependent process (Johnson et al., 2006; Johnson and Jackson, 2008; Randolph et al., 2005). Two studies that conducted a limited amount of ultrastructural analysis using single-section TEM suggested a paracellular mode of diapedesis across afferent lymphatic vessels (Baluk et al., 2007; Stoitzner et al., 2002). However, it is clear that the overall roles of paracellular and transcellular pathways in this setting remain an open question (Johnson and Jackson, 2008).

In contrast to the afferent lymphatic vessels, studies that are much more extensive [often employing serial-section TEM and three-dimensional (3D) modeling] have been conducted to investigate diapedesis associated with other lymphatic structures. It has been shown in a largely conclusive manner that transcellular diapedesis predominates in the lymphatic sinuses of the lymph node (Farr et al., 1980; Olah and Glick, 1985) and various absorbing peripheral lymphatic vessels, under both normal and inflammatory conditions (Azzali, 1990; Azzali, 1998; Azzali, 2007a; Azzali and Arcari, 2000; Azzali et al., 1990a; Azzali et al., 1990b).

Transcellular diapedesis in peripheral-tissue inflammation

In general, inflammation is associated with cytokine-driven upregulation of adhesion molecules (such as E-selectin, ICAM1, VCAM1 and MAdCAM1) and with chemoattractant presentation by endothelia, leading to localized recruitment of leukocytes to the affected tissues. However, in vivo inflammatory responses vary greatly according to the specific tissue (or vascular bed), stimuli (e.g. pathogen infection, allergens, tissue trauma, ischemia, thrombotic events, and so on) and the resulting leukocyte subtypes that are recruited. Given such heterogeneity, it is difficult to make generalizations about the importance of paracellular versus transcellular diapedesis routes associated with peripheral-tissue inflammation.

The earliest studies to directly examine the route of extravasation during inflammation provided modest support for, at least partial, usage of a transcellular pathway by neutrophils in mechanically traumatized rat mesentery (Marchesi, 1961; Marchesi and Florey, 1960) and in ischemic dog pancreas (Williamson and Grisham, 1960; Williamson and Grisham, 1961). Although many subsequent studies (not reviewed here) suggested that paracellular diapedesis occurred exclusively, others provided further support for transcellular diapedesis of both neutrophils and lymphocytes in various tissues (skin, liver, lung, spleen, kidney and heart) in response to a range of inflammatory mediators [complement component 5a (C5a), neutrophil-activating peptide, interleukin (IL)1α, IL2 and leukotriene B4] (Fujita et al., 1991; Schubert et al., 1989). However, it was recognized that, in all of these single-section TEM studies, the route of diapedesis could not be conclusively determined.

The first unequivocal demonstration of inflammatory transcellular diapedesis was provided by Dvorak and co-workers, who demonstrated (using ultra-thin serial-section TEM) almost exclusive transcellular diapedesis of neutrophils in response to intradermal

injection of the bacterial chemoattractant formyl-Met-Leu-Phe (fMLP) (Feng et al., 1998). Quantitatively similar, and largely conclusive, results were obtained in a subsequent study that used fMLP injected into skin (Hoshi and Ushiki, 1999). Recently, Kubes and co-workers were the first to use serial-section confocal fluorescence microscopy to demonstrate unambiguous transcellular diapedesis of ~15% of neutrophils in post-capillary venules of macrophage inflammatory protein 2 (MIP2)-stimulated mouse cremaster muscle (Phillipson et al., 2006).

Transcellular diapedesis across the BBB and BRB during inflammatory pathology

The central nervous system (CNS) and retinal parenchyma are immune-privileged compartments in which leukocyte trafficking is tightly limited by the specialized endothelial blood-brain barrier (BBB) and blood-retinal barrier (BRB), respectively. A hallmark of the BBB and BRB is the exceptionally well-organized interendothelial tight junctions, which greatly limit paracellular permeability (Lightman and Greenwood, 1992; Rubin and Staddon, 1999). During inflammatory pathology of the CNS and retina, trafficking of leukocytes across the BBB and BRB is significantly upregulated. In such settings, many studies have documented a predominant role for transcellular diapedesis pathways (Table 1).

Although diapedesis across the BBB was initially thought to be paracellular in rat models of multiple sclerosis [in which experimental autoimmune encephalomyelitis (EAE) was induced] (Lampert, 1967), many subsequent studies have strongly supported transcellular diapedesis of mononuclear inflammatory cells across the BBB (Lossinsky et al., 1989; Lossinsky et al., 1991; Raine et al., 1990; Wolburg et al., 2005). In a variety of other inflammatory settings, including acute inflammatory autoimmune neuritis (experimental allergenic neuritis) (Astrom et al., 1968), post-operative neural degeneration (Matthews and Kruger, 1973), corticectomy-induced thalamic nerve degeneration (Barron et al., 1974) and acute inflammatory meningitis, lymphocytes and neutrophils both crossed the BBB largely via transcellular routes. Similarly, in an experimental model of posterior uveitis [experimental autoimmune uveoretinitis (EAU)] in rat and in IL1β-mediated acute inflammation in rat, lymphocytes, granulocytes and monocytes were shown to cross the BRB exclusively by a transcellular pathway (Bamforth et al., 1997; Greenwood et al., 1994). Interestingly, several authors have noted that, in the BBB or BRB, leukocytes seem to initiate diapedesis by ‘probing’ into endothelial cells with micron-scale, actin-rich ‘pseudopodia’ or ‘processes’ (Bamforth et al., 1997; Greenwood et al., 1994; Lossinsky et al., 1991; Wolburg et al., 2005) that, morphologically, are not unlike invadosomes (discussed below).

The studies summarized above provide extensive demonstrations of transcellular diapedesis in vivo in essentially all tissues in which diapedesis has been examined, although the extent to which paracellular and transcellular routes dominate varies greatly. It is difficult to infer mechanisms for transcellular diapedesis from these (largely static and descriptive) studies alone. Nonetheless, when combined with recent in vitro studies, which are more dynamic and experimentally tractable, these in vivo observations provide significant support for roles of specific leukocyte protrusive structures in transcellular diapedesis, as discussed below.

In vitro studies of transcellular diapedesis

The development of techniques for the isolation and culture of primary endothelial-cell monolayers in the early 1970s (Gimbrone et al., 1974; Jaffe et al., 1973) facilitated in vitro investigations of diapedesis, which (through the use of single-section TEM and light microscopy) initially demonstrated diapedesis through paracellular pathways (Beesley et al., 1979; Burns et al., 2003; Furie et al., 1987; Luscinskas et al., 2002; Muller, 2001; Muller, 2003; Pawlowski et al., 1988). However, beginning in 2004, a growing collection of investigators started to make unambiguous observations of transcellular diapedesis in vitro, largely via the use of advanced fluorescence imaging approaches (Carman et al., 2007; Carman and Springer, 2004; Cinamon et al., 2004; Ferreira et al., 2005; Gerard et al., 2009; Keuschnigg et al., 2009; Marmon et al., 2008; Millan et al., 2006; Nieminen et al., 2006; Riethmuller et al., 2008; Yang et al., 2005). These observations have been made with a range of primary and transformed endothelial cells [human umbilical-vein endothelial cells (HUVECs), human coronary-artery endothelial cells (HCAECs), human dermal (HDMVECs) and lung (HLMVECs) microvascular endothelial cells, human lymphatic endothelial cells (HLyECs), and murine brain microvascular endothelial cells (bend.3)] and leukocytes (neutrophils, monocytes, and memory and effector lymphocytes). Moreover, these studies have included diverse migratory and inflammatory stimuli [namely, fMLP, IL1β, IL8, platelet-activating factor (PAF), stromal cell-derived factor-1 (SDF-1) and TNFα] and both static and physiologic shear-flow conditions. The quantitative contribution of the transcellular route in these studies ranged from ~5 to ~60% of the total diapedesis.

Mechanisms for transcellular diapedesis

Adhesion molecules

The cell-cell adhesion molecules involved in transcellular diapedesis have begun to be characterized. Several investigations show enrichment of endothelial ICAM1 on transcellular pores of endothelial cells and enrichment of the integrin LFA1 (the ICAM1 receptor) on the closely apposed pore-spanning segments of transmigrating leukocytes (Carman et al., 2007; Carman and Springer, 2004; Gerard et al., 2009; Millan et al., 2006; Yang et al., 2005). Numerous studies have also demonstrated so-called endothelial ‘docking structures’ or ‘transmigratory cups’ enriched in ICAM1 and VCAM1 that partially ‘embrace’ migrating leukocytes (Barreiro et al., 2002; Carman et al., 2003; Carman and Springer, 2004; Faustmann and Dermietzel, 1985; Fujita et al., 1991; Millan et al., 2006; Nieminen et al., 2006; Phillipson et al., 2008; Raine et al., 1990; Riethmuller et al., 2008; van Buul et al., 2007; Williamson and Grisham, 1961; Wolburg et al., 2005). These actin- and vimentin-dependent structures seem to have guidance or traction roles, and are important for both paracellular and transcellular diapedesis. Finally, a few studies have suggested that the ‘junctional’ adhesion molecules that are involved in paracellular diapedesis [PECAM-1 and JAM-1 (Luscinskas et al., 2002; Muller, 2003)] might also be involved in transcellular migration (Carman et al., 2007; Gerard et al., 2009; Mamdouh et al., 2003).

Integrin-ligand interactions have also been implicated in determining the preference of leukocytes for paracellular versus transcellular routes. However, consistent roles for integrins are not yet evident. In one in vivo model, knockout of the integrin Mac1 led to a large increase (~65% of all extravasations compared with ~15% for wild type) in transcellular migration (Phillipson et al., 2006). In addition, an in vitro study demonstrated that neutrophil transcellular migration was strongly favored by high endothelial ICAM1 expression in a manner largely dependent on LFA-1

expressed on neutrophils (Yang et al., 2005). In both cases, altered adhesion, leading to altered lateral migration (discussed below), was suggested to be the basis for the change in route, but through opposite mechanisms [that is, Yang et al. proposed that leukocyte-endothelium adhesion was increased, whereas Phillipson et al. proposed that adhesion was reduced (Yang et al., 2005; Phillipson et al., 2006)]. Interestingly, a reduction of lymphocyte lateral migration in the absence of measurable changes in strength of adhesion (which was achieved by deletion of the Rac1 activator Tiam1) was also found to dramatically upregulate transcellular diapedesis (Gerard et al., 2009). Importantly, transcellular migration events tend to occur near junctions (discussed below), such that they often might not be adequately distinguished from paracellular migration events using light microscopy (Carman and Springer, 2008). Thus, the above studies might also reflect changes in location of transcellular diapedesis events (that is, relatively central versus peri-junctional transcellular diapedesis) (Carman and Springer, 2008).

Lateral migration

During extravasation, initial adhesion of leukocytes to the endothelium is followed by polarization and integrin-dependent lateral migration (Phillipson et al., 2006; Schenkel et al., 2004). This has been generally interpreted as a mechanism to allow leukocytes to move towards endothelial-cell junctions to enable paracellular diapedesis (Phillipson et al., 2006; Schenkel et al., 2004). However, lateral migration also precedes transcellular diapedesis (Carman et al., 2007; Cinamon et al., 2004; Millan et al., 2006; Yang et al., 2005) and probably has an analogous role in positioning leukocytes optimally (that is, at sites where transcellular pore formation can occur most efficiently). A wide range of in vivo EM studies suggest that transcellular diapedesis occurs preferentially in close juxtaposition to intact intercellular junctions (peri-junctionally) (Azzali, 1990; Azzali, 1998; Azzali, 2007a; Azzali and Arcari, 2000; Azzali et al., 2008; Azzali et al., 1990a; Azzali et al., 1990b; Bamforth et al., 1997; Campbell, 1972; Chamberlain and Lichtman, 1978; Cho and De Bruyn, 1981; Cho and De Bruyn, 1986; De Bruyn et al., 1971; Farr et al., 1980; Faustmann and Dermietzel, 1985; Feng et al., 1998; Greenwood et al., 1994; Lossinsky et al., 1989; Lossinsky et al., 1991; Marchesi and Florey, 1960; Marchesi and Gowans, 1964; Wolburg et al., 2005; Wolosewick, 1984) and it has been suggested that leukocytes therefore must somehow 'seek out these regions' (Campbell, 1972). This begs the question of how, in the absence of a discrete pre-existing locus (such as endothelial-cell junctions for paracellular diapedesis), such sites for transcellular pore formation (whether peri-junctional or otherwise) can be identified by the cell.

Probing by ILPs

As discussed below, in vitro studies suggest that ILPs – which are leukocyte structures that are similar to both podosomes and invadopodia (collectively subtended under the term 'invadosomes') – are crucial for transcellular diapedesis (Carman et al., 2007). Invadosomes are classically defined as actin-dependent adhesive and/or protrusive structures (~500 nm in both diameter and depth) that form specifically on the ventral surface of highly migratory and invasive cells, such as leukocytes, endothelial cells and transformed tumor cells (Gimona et al., 2008; Linder, 2009). Although the protrusive structures that are observed in the setting of leukocyte diapedesis (see below) share many features of classical invadosomes, other properties are unique or remain undefined

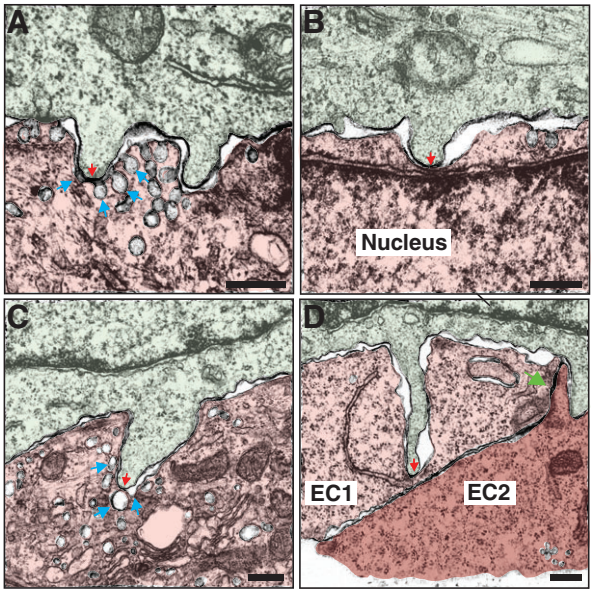

Fig. 3. Ultrastructure of leukocyte ILPs formed on endothelium. Shown are transmission electron micrographs of primary effector lymphocytes (green) migrating on activated microvascular endothelial cells (pink) as described (Carman et al., 2007). (A) Formation of typical ILPs on the endothelial-cell surface. (B) A shallow ILP being 'frustrated' by the relatively rigid nuclear lamina of the endothelial cell. (C) A more extended ILP than those shown in A. (D) An ILP that has nearly crossed an individual endothelial cell. Note that this event takes place in close juxtaposition to an intact adherens junction (green arrow) formed between two endothelial cells (EC1 and EC2). Blue arrows (A,C) highlight enrichment and plasma-membrane fusion of vesicles, VVOs and/or caveolae. Red arrows (A-D) emphasize regions of exceptionally close membrane-membrane contact that form at the tips of ILPs between lymphocytes and endothelial cells. Scale bars: 300 nm.

(Box 1). Thus, we refer to these as 'invadosome-like protrusions' (ILPs) to explicitly denote the current uncertainty of their precise relationship to the invadosomes that have been defined in other settings.

Live-cell fluorescence imaging studies have recently revealed that lymphocytes and monocytes dynamically protrude and retract (with half-lives of ~20 seconds) many ILPs (between 10 and 100) into the surface of the endothelium as they migrate laterally over it (Carman et al., 2007) (Fig. 2). These protrusions cause the formation of endothelial cell-surface invaginations (termed 'podoprints') (Carman et al., 2007). Studies using live-cell total internal reflection fluorescence (TIRF) microscopy demonstrate the formation of similarly dynamic lymphocyte-induced endothelial invaginations (Millan et al., 2006). TEM analysis demonstrated a continuum of protrusion depths (ranging from ~100 to ~2000 nm) (Fig. 3), and some of these structures spanned nearly the entire endothelial-cell depth, placing the apical and basal membranes in close apposition (Carman et al., 2007; Gerard et al., 2009) (Fig. 3D). Similar protrusions were also observed in TEM studies of neutrophil diapedesis (Cinamon et al., 2004). Importantly, such dynamic protrusive behavior was consistently seen to precede, and was functionally required for, efficient transcellular diapedesis (Carman et al., 2007; Gerard et al., 2009). On the basis of these investigations, a model has emerged in which dynamic ILPs 'probe' the endothelial surface during lateral migration as a means of stochastically identifying locations of relatively low endothelial resistance, into which protrusions can then extend progressively to promote

Box 1. How do diapedesis-associated ILPs relate to 'classical' invadosomes?

Morphology

- Invadosomes have width and depth dimensions ranging from ~0.5 to several μm
- ILPs range from ~0.1 to ~2 μm in width and depth

Molecular composition

- Invadosomes have an actin- and cortactin-rich core that is surrounded by peripheral integrin, talin and vinculin. Additionally, many other key regulatory and signaling proteins are typically associated with invadosomes, such as Arp2/3, WASP, N-WASP, Src and RhoA
- ILPs have an actin-rich core and peripheral integrin, talin and vinculin. Other markers remain to be investigated. Although the localization of WASP at ILPs has not been characterized, ILPs depend on functional WASP

Substrate

- Invadosomes have classically been assessed on solid two-dimensional (2D) substrates (glass or plastic), 2D matrix or on 2D bone (for osteoclasts). Although invadosome formation and function in 3D matrices is thought to be physiologically relevant, invadosomes have not yet been thoroughly assessed in such settings
- ILPs have only been observed in the context of cell-cell interactions. The disparity in substrate settings in which invadosomes and ILPs have been observed represents a major challenge to understanding their relationship to each other

Function

- Invadosomes in general are known to have roles in adhesion, migration and matrix degradation. Specialized podosome arrays ('podosome belts') of osteoclasts facilitate bone resorption. Matrix invasion is generally thought of as a specialization of invadopodia
- ILPs seem to function in probing the endothelial surface and in transcellular diapedesis (clearly an invasive property). On physiological endothelial substrates, lateral migration and adhesion do not seem to require ILPs

Dynamics

- The lifetime of invadosomes varies greatly from tens of seconds (e.g. macrophage podosomes) to tens of minutes (e.g. osteoclast podosomes or transformed-cell invadopodia)
- ILPs have lifetimes from tens of seconds to several minutes

Protrusiveness

- Invadosomes are variably protrusive: whereas invadopodia are generally thought to be protrusive, it remains unclear whether podosomes are necessarily so
- ILPs are defined, in part, by their protrusion into cellular substrates

Degradation of the extracellular matrix

- Matrix degradation by invadosomes, specifically at sites of active actin polymerization, has been proposed as a defining characteristic for these structures
- ILPs have yet to be assessed for either degradative activity with respect to the matrix (or other proteins) or for protease enrichment or secretion. There is no obvious requirement for proteases in formal transcellular pore formation – but, if they were present, ILP-localized proteases might alter local adhesion-molecule and chemokine densities on the endothelium, thereby changing adhesion and signaling dynamics. Alternatively, such proteases might have a role in basement-membrane degradation

transcellular pore formation. Interestingly, very recent studies have characterized lymphocyte structures termed 'invasive filopodia' that are highly analogous to ILPs (in terms of dynamics, molecular composition and morphology), and that were similarly ascribed a role in probing for sites for diapedesis, although not explicitly by the transcellular route (Shulman et al., 2009).

Importantly, this ILP probing model in its basic form has been previously suggested and supported by diverse observations. Indeed, the first description of a podosome (and use of this term) was made in the context of transcellular migration of lymphocytes and eosinophils across bone-marrow endothelium in vivo (Wolosewick, 1984). The authors predicted that the protrusive force supplied by such actin-enriched structures might drive transcellular pore formation. Shortly thereafter, Marchisio and co-workers conducted the first detailed molecular characterization of podosomes in transformed fibroblasts (Tarone et al., 1985). Subsequently, the same group showed that leukocytes (natural killer cells) that adhered to endothelium in vitro formed podosomes, and suggested that these functioned in endothelial penetration during diapedesis (Allavena et al., 1991). Finally, although the structures that were visualized have been referred to with various terms, including 'microvillus-, filopodium- and finger-like protrusions', 'processes', 'pseudopodia' and 'probing pseudopods', many in vivo studies of transcellular diapedesis have also demonstrated the endothelium-directed protrusion of structures with clearly invadosome-like morphology from lymphocytes, monocytes, neutrophils, eosinophils and acute myeloid leukemia tumor cells (Astrom et al., 1968; Azzali et al., 2008; Bamforth et al., 1997; Barron et al., 1974; Becker and De Bruyn, 1976; De Bruyn et al., 1989; De Bruyn et al., 1971; Farr and De Bruyn, 1975; Faustmann and Dermietzel, 1985; Feng et al., 1998; Fujita et al., 1991; Greenwood et al., 1994; Lossinsky et al., 1989; Lossinsky et al., 1991; Lossinsky and Shivers, 2004; Marchesi and Florey, 1960; Matthews and Kruger, 1973; Olah

and Glick, 1985; Raine et al., 1990; Williamson and Grisham, 1960; Wolburg et al., 2005; Wolosewick, 1984) (Table 1). The actions of these protrusions have often been interpreted as 'probing' the endothelium and potentially driving transcellular pore formation (Astrom et al., 1968; Bamforth et al., 1997; Farr and De Bruyn, 1975; Greenwood et al., 1994; Lossinsky et al., 1989; Lossinsky et al., 1991; Lossinsky and Shivers, 2004; Marchesi and Florey, 1960; Olah and Glick, 1985; Raine et al., 1990; Wolburg et al., 2005; Wolosewick, 1984). Thus, the ability and tendency of leukocytes to extend ILPs into the endothelial surface during diapedesis seems to be broadly relevant.

Mechanotransduction by ILPs?

An implicit component of the model presented above is that ILPs can somehow sense mechanical resistance and, in response, alter their signaling and dynamics. Although this has not been widely investigated, at least two recent studies demonstrate that podosomes can serve as 'dynamic mechanosensors' (Collin et al., 2008; Collin et al., 2006). In these two studies, using polyacrylamide collagen-coated substrates of defined rigidity, Collin et al. demonstrated that the lifespan and density of fibroblast podosomes depended on substrate flexibility and that such mechanosensory properties were mediated in part through myosin-II motor proteins. These observations support the plausibility of a model in which mechanical probing of the endothelial surface by ILPs transduces inside-out and outside-in signals that regulate ILP activity and ultimately influence the efficiency of transcellular diapedesis.

Endothelial membrane fusion and pore formation in response to ILPs

To accomplish pore formation, transcellular diapedesis requires that energy-dependent membrane-fusion events occur. The protrusive forces that are supplied by ILPs clearly promote close apposition of apical and basal endothelial membranes (Fig. 3D), and might also supply energy that is directly involved in the formal membrane-fusion process. In addition, studies suggest that ILPs somehow trigger specific proactive responses in the endothelium that facilitate transcellular pore formation. In vitro studies show enrichment of the caveolar marker caveolin-1, plasmalemma vesicle associated protein-1 (PV-1), various vesicles and vesiculo-vacuolar organelles (VVOs) and fusogenic proteins (namely, the SNAREs VAMP2 and VAMP3) in the endothelium at sites of ILP protrusion and transcellular pore formation (Carman et al., 2007; Keuschnigg et al., 2009; Migliorisi et al., 1987; Millan et al., 2006) (Fig. 2; Fig. 3A,C). Similar observations have been made in vivo (Carman et al., 2007; De Bruyn et al., 1989; Greenwood et al., 1994; Olah and Glick, 1985), leading one of the research groups to speculate that "the fusing of the vesicles may form a gradual trans-endothelial channel in which the pseudopod of the lymphocyte penetrates and by this mechanism the lymphocyte may cross the endothelium" (Olah and Glick, 1985) (Fig. 2). Vesicle fusion might also facilitate local delivery of adhesion receptors (Mamdouh et al., 2003; Millan et al., 2006) and chemokines (Middleton et al., 2002) that might modify ILP activity and the efficiency of transcellular migration. The functional significance of vesicle-fusion activity was suggested by studies in which short interfering RNA (siRNA)-mediated knockdown of caveolin-1 and pharmacologic perturbation of the soluble N-ethylmaleimide-sensitive factor (NSF)-attachment protein (SNAP)-SNARE membrane-fusogenic machinery in endothelium significantly reduced the efficiency of transcellular diapedesis (Carman et al., 2007; Millan et al., 2006). However, much more detailed characterization of the molecular mechanisms by which ILPs 'trigger' such responses, and of the consequences of this triggering activity, is still required.

Other settings for transcellular diapedesis

Crossing epithelial and other cell types

The transcellular mode of cell migration might also be used to cross non-endothelial barriers. For example, it has been demonstrated in vivo that neutrophils can migrate transcellularly through the pericytes that underlie the vascular endothelium (Feng et al., 1998). In addition, extensive migration of leukocytes across epithelial cell layers (e.g. the mucosal epithelia of the intestine, airway and urinary tract) occurs in vivo (Zen and Parkos, 2003). Routes and mechanisms for this migration remain either only partially understood or uninvestigated (Zen and Parkos, 2003). One recent study demonstrated that paracellular diapedesis of neutrophils occurs in an in vitro model system (Porter et al., 2008). In an alternative (albeit less physiologic) system, we recently found that lymphocytes could readily form transcellular pores across epithelial cells (in particular, CHO-K1 cells expressing GFP-tagged ICAM1) by means of probing by ILPs (Carman et al., 2007).

Diapedesis of non-leukocyte cell types

Transcellular diapedesis might also be used by non-leukocyte cell types. For example, a variety of studies have observed reticulocytes performing transcellular intravasation and have observed megakaryocytes forming long transcellular processes (which might be important for the release of platelets into the bloodstream), both across bone-marrow endothelium (Becker and De Bruyn, 1976; Campbell, 1972; Chamberlain and Lichtman, 1978; De Bruyn et al., 1971; Muto, 1976). In addition, several in vivo studies provide strong support for the use of a transcellular mechanism during diapedesis of certain metastatic tumor cell types (Azzali, 2006; Azzali, 2007b; De Bruyn et al., 1989). Finally, stem cells, which are important emerging cellular therapeutics, clearly undergo extensive trafficking in vivo (Chamberlain et al., 2007; Laird et al., 2008). The routes and mechanisms used by these cells for diapedesis await characterization. The degree to which mechanisms, including ILP probing, that are used by leukocytes will be relevant to other cell types during transcellular diapedesis remains an open question.

Other possible roles for ILP probing

Crossing the basement membrane?

In both intravasation and extravasation, the endothelial barrier is only one of the key obstacles to be overcome. The basement membrane also provides a formidable and often rate-limiting barrier. Precisely how the basement membrane is crossed by leukocytes remains poorly defined. Some studies have suggested that proteolytic degradation of the basement membrane enables the passage of leukocytes. This idea is well supported, at least for metastatic tumor cells during their intravasation across lymphatic endothelial layers (Gimona et al., 2008). It seems conceivable that, if proteases are enriched in leukocyte ILPs as they are in classical invadosomes (Chavrier, 2009), this could facilitate basement-membrane degradation and crossing. However, the requirement for basement-membrane degradation during diapedesis remains somewhat controversial, and several non-proteolytic modes of basement-membrane transmigration have been hypothesized (Rowe and Weiss, 2008). In this vein, it is interesting to note that neutrophils extravasating in vivo have been observed to transmigrate

preferentially at pre-existing sites of relatively attenuated basement membrane (Wang et al., 2006). By analogy with their roles in probing for attenuated regions of endothelium, ILPs might act similarly in locating regions of basement membrane that are permissive for migration.

Interrogating the endothelial surface biochemically?

In essence, the pathfinding model discussed above posits that ILPs are sensory probes and organelles, as well as invasive structures. The observations made thus far focus on a putative role for ILPs in sensing the biomechanical properties of cellular, and possibly matrix, substrates (i.e. a mechanotransduction role). It is interesting, however, to consider whether such a 'probing' function might also include biochemical sensing of the local environment.

The surface of all cells is modified by a gel-like polysaccharide coating that is termed the glycocalyx, which is made up of diverse proteoglycans and glycoproteins (Reitsma et al., 2007; Weinbaum et al., 2007). On a typical cell, the glycocalyx is at least 45-nm thick, but it can be substantially thicker (up to ~500 nm) in some cases, such as on the lumen-facing surface of endothelial cells (Reitsma et al., 2007; Weinbaum et al., 2007). The glycocalyx can serve as a reservoir of non-covalently immobilized chemokines and other chemoattractants (Middleton et al., 2002; Proudfoot et al., 2003). In addition, the glycocalyx provides a formidable energy barrier (by means of both steric and electrostatic repulsion) to close membrane-membrane encounter between cells, such that relatively small cell-surface adhesion and signaling molecules are effectively shielded (Bell, 1978; Bell et al., 1984; Reitsma et al., 2007; Springer, 1990; Weinbaum et al., 2007). Forces provided by ILPs seem to provide sufficient energy to overcome this barrier, driving close membrane-membrane apposition (see Fig. 3, red arrows) and thereby promoting molecular interactions that might otherwise be inefficient or impossible. Indeed, our recent studies suggest that recognition of endothelial major histocompatibility complex (MHC)-antigen complexes by T-cell receptors on lymphocytes is dependent on ILPs (Peter T. Sage and C.V.C., unpublished observations). Thus, ILPs might facilitate a kind of informational scanning of local membrane surfaces, literally allowing cells to get a 'deeper' understanding of their local environment.

Conclusions and perspectives

The significant number of studies in vivo, along with emerging in vitro studies, demonstrates that two physiologically relevant pathways (paracellular and transcellular) for leukocyte diapedesis coexist, with greatly varied utilization of each in distinct settings. The cellular and molecular determinants of the observed route preferences represent a crucial unresolved issue. In contrast to the extensively studied paracellular pathway, mechanisms for transcellular diapedesis are just beginning to be elucidated, and point towards significant roles for leukocyte ILPs. These seem to allow for a kind of 'migratory pathfinding' in which stochastic 'probing' of the endothelial cell surface is coupled to progressive ILP invasion at sites that are permissive for transcellular pore formation. Permissive sites might include not only endothelial regions in which the cell is sufficiently attenuated (that is, sufficiently thin and/or devoid of resistance-producing intracellular components, such as nuclear lamina or other regions dense in cytoskeletal fibers), but also regions that are responsive to ILP-mediated 'triggering' of fusogenic activity in the endothelium. Many important questions regarding the molecular regulation of ILPs and the precise relationship of these structures to 'classical' invadosomes remain to be addressed. It is particularly important to resolve whether ILPs exhibit localized proteolytic activity as invadosomes do and, if so, what roles this has in transcellular diapedesis. Finally, the degree to which the 'probing activity' of ILPs has a role in pathfinding in other settings, and the issue of whether ILPs have additional 'probing' functions (as suggested above), will be important additional future areas of inquiry.

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